EXHIBIT N

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COMMENTARY

Do dose response thresholds exist for genotoxic alkylating agents?

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The demonstration and acceptance of dose response thresholds for genotoxins may have substantial implications for the setting of safe exposure levels. Here we test the hypothesis that direct-acting DNA reactive agents may exhibit thresholded dose responses. We examine the potential mechanisms involved in such thresholded responses, particularly in relation to those of alkylating agents. As alkylating agents are representative model DNA reactive compounds with well characterized activities and DNA targets, they could help shed light on the general mechanisms involved in thresholded dose responses for genotoxins. Presently, thresholds have mainly been described for agents with non-DNA targets. We pay particular attention here to the contribution of DNA repair to genotoxic thresholds. A review of the literature shows that limited threshold data for alkylating agents are currently available, but the contribution of DNA repair in thresholded dose responses is suggested by several studies. The existence of genotoxic thresholds for alkylating agents methylmethanesulfonate is also supported here by data from our laboratory. Overall, it is clear that different endpoints induced by the same alkylator, can possess different dose response characteristics. This may have an impact on the setting of safe exposure levels for such agents. The limited information available concerning the dose response relationships of alkylators can nevertheless lead to the design of experiments to investigate the mechanisms that may be involved in threshold responses. Through using paired alkylators inducing different lesions, repaired by different pathways, insights into the processes involved in genotoxic thresholds may be elucidated. Furthermore, as alkyl-guanine-DNA transferase, base excision repair and mismatch repair appear to contribute to genotoxic thresholds for alkylators, cells deficient in these repair processes may possess altered dose responses compared with wild-type cells and this approach may help understand the contribution of these repair pathways to the production of thresholds for genotoxic effects in general. Finally, genotoxic thresholds are currently being described for acute exposures to single agents *in vitro*, however, dose response data for chronic exposures to complex mixtures are, as yet, a long way off.

Introduction

During the safety evaluation of chemical compounds, the detection and quantification of any tissue specific effects are critical for the setting up of exposure standards. When toxic effects are detected, the exposure concentrations at which they occur and the types of dose response relationships observed, will represent important components in the regulatory process. However, regulatory action will be influenced by the type of response and whether there are concentrations below which a response is not observed, i.e. whether the response is thresholded. The European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) has defined an absolute toxicological threshold as 'a concentration below which a cell would not "notice" the presence of a chemical'. In this article we consider the evidence for potential thresholds of genotoxic alkylating agents.

As absolute thresholds are extremely difficult to assess, it is fair to say that the genotoxic thresholds alluded to here are more properly termed 'biological or practical thresholds'. Numerous types of thresholds have been described previously [absolute, biological, apparent, acceptable, statistical, no observable effect level (NOEL), real, alleged, etc.] these are reviewed elsewhere in the literature, to simplify things, we concentrate here on thresholds that can be observed experimentally. Thresholded responses have previously been reported for non-DNA reactive agents; here, we evaluate if thresholds exist for a group of DNA reactive agents. The concept of thresholds of activity of DNA reactive genotoxic agents is currently an important area of concern for toxicologists. Previously, DNA-reactive genotoxins have been assumed to have linear dose responses, i.e. non-thresholded.

In terms of genetic toxicology, the main endpoints analysed for thresholded dose responses, have been DNA adduct formation, gene mutation, and chromosomal aberration. Here, we are concerned with evaluating the available data for genotoxic thresholds induced by exposure of cells and whole organisms to exogenous chemical agents (particularly alkylating agents). These thresholds can often be described by the terms NOELs, or 'non-linear dose responses', although in the latter case non-linear responses are not always thresholded.

The demonstration and acceptance (by the scientific and regulatory community) of the concept of a threshold of genotoxic activity can have important economic implications for the use of individual compounds. The unambiguous demonstration of a threshold of genotoxic activity indicates that a compound will not produce mutations (or chromosomal effects) below a critical exposure level, thus reducing the potential for the induction of cancer or congenital abnormalities at these exposures. As it is difficult to experimentally assess absolute thresholds of activity (1), a more pragmatic approach relies on assessing the biological significance of thresholded agents, above the designated threshold dose (2). Therefore, thresholds can be considered to represent the separation between 'no biologically significant effects' and 'biologically significant effects'. Lowest observable effect levels (LOELs) represent

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the dose range of this transitionary stage. Statistical methods can then be used to confirm the existence of thresholds by identifying genuine increases in the endpoint at a specific dose, compared with the effects seen in the untreated control replicates, or between the levels of the endpoints at either side of the threshold dose. Importantly, the biological basis for the genotoxic threshold is also fundamental to understanding the mechanisms involved and for accepting the plausibility that a threshold exists.

Table I displays examples of the potential mechanisms that might be involved in the formation of genotoxic thresholds. There are two main mechanisms which can lead to genotoxic thresholds; (i) involvement of redundant targets and (ii) the contribution of protective mechanisms (Figure 1). The first mechanism is responsible, for example, for the thresholds identified with spindle poisons (which, when damaged can lead to aneuploidy). In the thresholded dose region sufficient spindle fibres are poisoned to elicit abnormalities in chromosome segregation whereas, at lower doses, enough spindle fibres are intact to allow normal segregation (3). The second mechanism can involve detoxification of the genotoxic agent (4), exclusion from the cell/nucleus (5) or repair of the induced damage (6). It should be noted that there is a significant difference in the mechanisms involved in thresholds for non-DNA damaging agents (e.g. multiple targets) and DNA damaging agents (e.g. DNA repair).

Table I. Examples of mechanisms that may be responsible for genotoxic thresholds produced by alkylating agents

Targets/activity involved	Mechanism involved/consequence
Microtubules, other redundant cellular constituents	Need to poison/inactivate multiple targets before effect observed, e.g. spindle fibres
Membranes	Shielding/exclusion of chemical from cell/nucleus
Metabolic enzymes	Detoxification/conjugation of agents before interaction with DNA/other targets
DNA repair	Repair of damage before mutation/ chromosome abnormality induced
DNA redundancy	Only coding/regulatory DNA sequence damage lead to mutation
Apoptosis triggers	Damage eliminated by cell death, only when apoptosis is overcome is damage observed

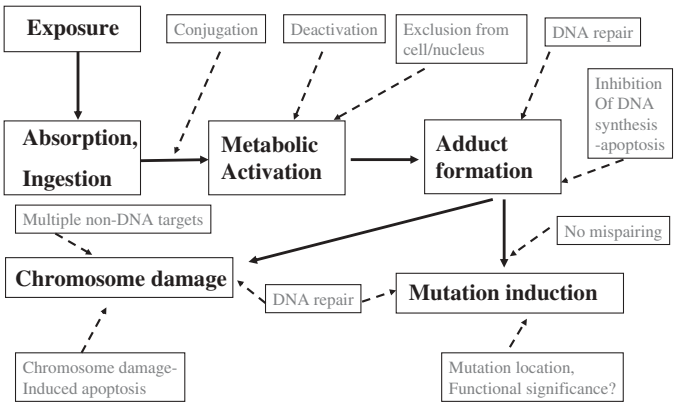

Fig. 1. Diagram showing the route and the potential mechanisms of genotoxic thresholds.

Although accepted on a theoretical basis, there has been little experimental evidence demonstrating thresholds for genotoxins (7–9). Even if thresholds can be experimentally demonstrated in a specific situation, it may be difficult to assign specific threshold doses for populations as a whole, owing to the heterogeneity of individual responses within a population (10).

The principal view of regulatory authorities over the past three decades has been that genotoxins exhibit a linear relationship between exposure dose and mutagenic response (11). This has been interpreted such that it can be assumed there is no safe level of DNA damage, however low this is within the DNA. To many people, however, thresholds (or NOELs) do exist and organisms can tolerate low levels of DNA damage. Indeed, these organisms exist in an environment which leads to low levels of DNA damage being induced, and they have evolved to cope with this. Hence, even though regulators are becoming more enlightened with regard to the existence of thresholds (11,12), the current viewpoint is that thresholds have to be proven on an 'agent by agent basis'. This point of view may well change as more data become available from chemicals with similar modes of action. It is again important to point out that DNA damaging agents and non-DNA damaging agents are mechanistically distinct and are likely to be treated differently in regulatory terms.

To demonstrate a thresholded response, the mechanism (target) should always be known. Furthermore, the difficulty of extrapolating from *in vitro* to *in vivo* or indeed between different *in vitro* cell models should be appreciated (7), as should the extrapolation from somatic cells to germ cells. At present, thresholds have only been assessed for single exposures to single agents, not complex mixtures which represent the main sources of human exposures (13). Mixtures of chemicals may show synergism of genotoxicity, or indeed antagonism of genotoxicity (14), which complicate matters further. Furthermore, chronic exposures have not yet been addressed in studies looking at dose response relationships.

The concept of a threshold will also vary depending upon the type of chemical, for example, the 'single hit' argument might be true for mutagenicity of a bulky adduct [e.g. aflatoxin B1 is thought to have no threshold (15)], but for smaller adduct types, it may not always be the case that a mutation is produced from every adduct. Hence, mispairing potential will also influence whether a linear or thresholded effect is seen (obviously repair, detoxification etc., can still impose a threshold for a mispairing adduct). Chemical agents that cause modification or inactivation of multiple cellular targets may exhibit a thresholded response. To date, experimental evidence of thresholded responses has only been demonstrated for spindle poisons, ionizing radiation, methylmethanesulfonate (MMS) and topoisomerase II inhibitors (7,9,16–18). Table II contains some examples where thresholds of genotoxicity have been described.

Mechanisms that may lead to threshold responses for DNA adduction and mutation

There is evidence from molecular dosimetry experiments that there is no threshold for DNA adduct formation and that adducts form in direct proportion to the exposure dose (19,20). From this, the 'single hit, single target' model would appear to be correct for DNA reactive agents which would show a linear dose response. However, there is also a counter-argument that

Table II. Examples of published data demonstrating genotoxic thresholds in other chemical (and physical) agents

Agent	Threshold?	Type of agent	Test used	Reference
Lindane, Malathion, Metacid[a]	Yes	Pesticides	Clastogenicity, dominant lethality	72
MeIQX	Yes	Cooked meat carcinogen	Lac I mutation, pre-neoplastic liver foci formation.	73
X-radiation[b]	Yes	Ionizing radiation	Drosophila somatic mutation analysis	74
PhIP	Yes	Cooked meat carcinogen	DNA adduction, aberrant crypt foci	75
Vinyl acetate	Yes	Industrial chemical	Various	Reviewed in 15
Etoposide, doxorubicin, genistein, ciprofloxacin	Yes	Topoisomerase II inhibitors	Micronucleus formation	7
Colchicine, carbendazim, mebendazole, nocodazole	Yes	Spindle poisons	Micronucleus formation	16,17

[a]Threshold differs depending on endpoint used.
[b]Threshold less evident in repair deficient cells.

even DNA adduct formation may be thresholded, as low doses of DNA reactive agents will be excluded from the nucleus by cell membranes and by interaction with cytoplasmic elements preventing access to the DNA. Furthermore, as sensitive adduct detection methods take no account of where in the genome the adducts are, there could be effective removal of the small percentage of coding region adducts, but persistence of the intergenic adducts, which comprise the bulk of the total adducts formed. A linear relationship between exposure and dose does not exist for agents that need to be metabolized to form DNA reactive species or for those that react with multiple cellular targets. Indeed, DNA adducts may be subsequently removed by repair mechanisms imposing a threshold post adduction. Zito (20) claims that DNA repair will not impose a genotoxic threshold, but will merely 'only change the slope and not the shape of the dose response'. This remains to be seen, as no experimental data are yet available to confirm or contradict this, although changing the slope of the dose response in some dose regions may ultimately alter the shape too. Conversely, it is accepted that genotoxins that augment existing background levels of damage may cause a linear response (21). An example here would be an agent that elevates the endogenous levels of oxidative damage (8).

Although there may be a linear relationship between exposure and DNA adduct formation, it is certainly true that 'not all adducts are created equal'. In terms of mutation induction, the important DNA adducts are those which:

(i) avoid immediate repair
(ii) mispair during replication
(iii) are positioned within an exon (or a gene regulatory sequence, or intronic bases involved in splicing) thus giving rise to an amino acid change (or change in expression efficiency).

Furthermore, the 'biological significance' of adduct formation has not yet been established due to the fact that methods for measuring mutations (a major biologically significant result of DNA adduction) are not as sensitive as those measuring DNA adducts (20). DNA adducts can now be detected at frequencies of 10^{-10} (22), whereas mutations are only generally detectable at frequencies of $10^{-5}/10^{-6}$ (23–25). The formation of DNA adducts may not always be thresholded, but the conversion of these adducts into biologically relevant mutations may exhibit a threshold effect. This effect is a consequence of the saturation of DNA adduct repair processes and of the misreplication potential of the adduct. Due to the fact that adduct detection methods are now able to detect adducts at extremely low frequencies, it is now difficult to separate adducts formed by chemical exposures from those produced endogenously. For example, Swenberg *et al*. (22) showed that prolonged exposure to vinyl chloride increased adduct levels by only 2-fold, compared with those of non-exposed samples. The superior sensitivity of adduct detection methods may lead to questions regarding the relevance of adduct data in assessing genotoxic risk for two reasons. Firstly, as mentioned above, it is now sometimes impossible to separate exogenous adducts from those formed endogenously. Secondly, the biological significance of adduct formation is unclear because they may not always lead to mutations (or may be present in intergenic sequences) and hence may be tolerated by cells in many instances. Nevertheless, DNA adduct formation is a robust reliable biomarker of exposure, that can easily be quantified and is undoubtedly linked to overall carcinogenic risk.

The sensitivity of the methods used to study genetic endpoint dose responses can have a major bearing on the interpretation of the data. As mentioned above, DNA adduct measurements are now so sensitive that background levels of adducts can be measured in human tissues. The sensitivity of methods for measuring mutation or chromosome damage are well below that for adducts. This is particularly true for point mutations. The lack of sensitivity of this begs the question: what happens below these detection limits? It is possible that there are non-linear effects below the detection limit that we are currently missing, or conversely, that detected thresholds are a consequence of the insensitivity of the methods used at low doses. Further developments are required to produce more sensitive methods for detecting rare DNA mutations.

Mechanisms leading to thresholds for chromosomal abnormalities

A commonly identified form of damage induced by either direct or indirect genotoxic chemicals is the production of chromosomal aberrations. DNA damaging agents may produce DNA adducts that are subsequently misrepaired resulting in strand breakages, or the chemicals may directly induce chromosomal breaks. Non-DNA damaging substances may target the mechanical components required for chromosome segregation (e.g. microtubules, kinetochores, centrioles), DNA synthesis (e.g. topoisomerases, DNA polymerases, imbalanced nucleotide pools) or DNA repair enzymes (e.g. polymerases, endonucleases, ligases), which subsequently give rise to structural and numerical chromosomal abnormalities (4,13).

Chromosomal damage can be assessed by the cytogenetic analysis of chromosomes at metaphase. However, this is a laborious and time consuming process and is prone to

artifactual loss of chromosomes during slide preparation. Currently, a frequently used technique for the rapid identification of chromosome damage following exposure to genotoxic chemicals is a combination of the cytochalasin B blocked micronucleus assay (26) with kinetochore labelling to discriminate between aneugenic and clastogenic events. This assay is highly sensitive and holds strong statistical power because a large number of individual cells can be rapidly scored; hence, it is ideally suited for assessing the effects of exposure to very low concentrations of genotoxins as required for examining whether a threshold for chromosome damage is present.

Chromosome segregation during mitosis is a multi-factorial event involving a large number of components required for the mechanical distribution of the chromosomes, together with signal transduction pathways that monitor the whole process. An indirect acting genotoxin would therefore have to damage multiple targets before a biological effect such as non-disjunction occurs (13). For example, spindle poisons such as nocodazole or colchicine will only induce non-disjunction at a concentration that damages enough tubulin molecules to disrupt spindle formation (17). Thus, non-DNA targeting agents are likely to demonstrate a threshold response and evidence to support this is now accumulating (Table II). Using the micronucleus assay in conjunction with fluorescent *in situ* hybridization (FISH), potential thresholds have been established for the aneugens colchicine, mebendazole, nocodazole, nitrobenzene, benzonitrile and benomyl and its major active metabolite carbendazim, all of which are spindle inhibitors (16,27–29).

Aims of this commentary

As genotoxic thresholds have only been established for a limited number of agents (mostly non-DNA damaging aneugens), we aimed in this commentary, to identify and assess the available data on the existence of, and mechanistic basis for, genotoxic thresholds for DNA reactive alkylating agents. Alkylating agents are an important class of genotoxins, whose mechanisms of action and DNA targets are well characterized. Data obtained on the dose response relationships of alkylators could help the understanding of the concept of genotoxic thresholds in general. Indeed, the data available for genotoxic thresholds for alkylators could well be extrapolated to other agents with similar mechanisms of action and/or DNA targets.

We have assessed here the published data for thresholds in terms of mutation induction and the induction of chromosomal abnormalities following exposure to a range of alkylators. We have specifically examined the available evidence for threshold effects for ethylnitrosourea (ENU), methylnitrosourea (MNU), ethylmethanesulfonate (EMS) and MMS, although evidence of thresholds for all alkylating agents were sought. These four alkylators were focused on, as they form two very interesting pairs of alkylators, with differing mechanisms and DNA repair pathways. The analysis of the different mechanisms of action of these alkylating agents may lead to insights and testable predictions about the likelihood of thresholds being determined for these agents. In particular, we have evaluated the contribution of DNA repair to genotoxic thresholds of alkylating agents; however, we accept that other processes such as detoxification, conjugation, exclusion, etc. may also contribute to this process.

Alkylating agents

Alkylating agents are electrophilic compounds with the ability to attack the nucleophilic centres of DNA (30,31). There are many species of alkylating agents, some monofunctional, some bifunctional (i.e. single- or double-reactive groups). The bifunctional alkylating agents (nitrogen mustards, etc.) represent cross-linking agents and as such, are effective at preventing DNA replication, hence their use as chemotherapeutic agents. Exposure to alkylating agents can occur through occupational exposures, cigarette smoke, environmental exposure (in food, detergents, cosmetics, the atmosphere, etc.) and through endogenous nitrosation of secondary amines (32). Indeed, ENU has been shown to be formed in the human gut by nitrosation processes (33).

There are many centres in double-stranded DNA which are targeted by alkylating agents, the target types and the relative reactivity depend upon individual alkylating agents. Table III shows a list of the alkylation sites in DNA induced by ENU, EMS, diethylsulfate, MMS, MNU and MNNG (methyl-*N*′-nitro-*N*-nitrosoguanidine). Figure 2 shows the locations of these adduct sites within double-stranded DNA. Most alkylating agents target N7G and O^6G and induce GC to AT mutations preferentially. ENU is an exception in that it induces 5- to

Table III. Adduct profile data for six different alkylators, demonstrating the different specificities of the alkylating agents in terms of DNA target

Adduct	ENU	EMS	DES	MMS	MNU	MNNG
s-value	0.26	0.67	0.64	>0.83	0.42	0.42
N3A	2.8–5.6	4.2–4.9	10	10.4–11.3	8–9	12
N7A	0.3–0.6	1.1–1.9	1.5	1.8	0.8–2	–
N3G	0.6–1.6	0.3–0.9	0.9	0.6	0.6–1.9	–
N7G	11–11.5	58–65	67	81–83	65–70	67
O^6G	7.8–9.5	2	0.2	0.3	5.9–8.2	7
N3C	0.2–0.6	0.4–0.6	0.7	<1	0.06–0.6	2
O^2C	2.7–2.8	0.3	Nd	Nd	0.1	–
N3T	0.8	Nd	Nd	0.1	0.1–0.3	–
O^2T	7.4–7.8	Nd	Nd	Nd	0.1–0.3	–
O^4T	1–2.5	Nd	Nd	Nd	0.1–0.7	–

Data extracted from (34). Not all adducts are shown, hence figures do not add up to 100%, e.g. ENU forms most of its DNA adducts against the sugar–phosphate backbone (>50%).
Nd, Not detectable; –, data not reported.

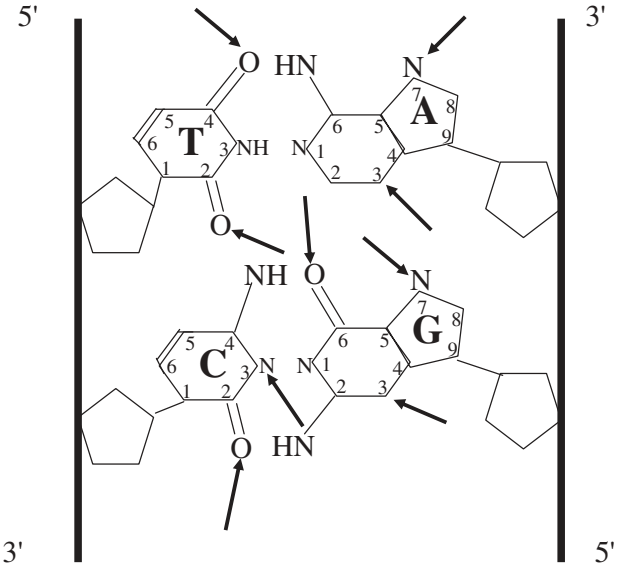

Fig. 2. Double-stranded DNA showing the sites of DNA adduct formation including the important targets for alkylators (N7G, N3A, O^6G, etc.).

8-fold less N7G than other alkylators, and preferentially targets thymine, causing relatively more AT mutations (31).

The reactivity of an alkylating agent is described by its Swain Scott Constant (*s*) (which ranges from 0 to 1). High *s*-value alkylating agents target nucleophilic centres such as N3A and N7G, low *s*-value alkylating agents target less nucleophilic centres such as O^6 guanine. Two types of alkylation reaction exist, either S_N1 or S_N2. S_N1 type reactions basically follow first order kinetics, whereas S_N2 type reactions involve an intermediate transition state. Low *s*-value alkylating agents tend to have S_N1 type kinetics, high *s*-value alkylating agents tend to follow S_N2 type kinetics (34). The alkylation pattern induced by specific alkylating agents leads to non-random adduct hotspots, which in turn lead to mutation hotspots upon misreplication (31). Alkylating agents in general target guanines preceded by a 5′ purine (35–37). The ability to repair damaged DNA is also an important factor in mutagenesis; indeed, observed mutation patterns reflect the influence of site-specific DNA adduct formation and DNA repair. In fact, it has been shown that DNA repair rates (of N7G in particular) vary across the genome and are DNA sequence dependent (38). Hence the final mutation pattern depends not just on global DNA repair, but also on the specificity of the DNA repair machinery.

Mutations induced by alkylating agents are also known to be subject to tissue specificity. Indeed, the tissue specificity of certain agents may well impose a threshold in some cases. For example, ENU is known to induce 5-fold fewer adducts in the testes compared with the liver of mouse (39) possibly as a consequence of the restricted access of ENU to the testis DNA. There are also adduct and mutational differences between tissues as a consequence of the relative repair capacities of different tissues, e.g. the liver has the greatest repair capacity (31,40) and hence its DNA carries the lowest number of repairable adducts. Therefore, dose responses may exhibit tissue specificity and hence in order to overcome some of these issues, *in vitro* studies are widely used.

DNA repair of alkylating agent-induced DNA damage/mutation

The repair of alkylated DNA is essential to prevent mutagenesis and this is especially true for the oxygen adducts which can mispair during replication. O^6-alkylguanine causes GC to AT transition mutations (41), O^4-alkylthymine causes AT to GC transition mutations (42) and O^2-alkylthymine causes AT to TA transversion mutations (43). N-alkylpurines (N7G, N3A) do not mispair during replication, but they are prone to spontaneously forming apurinic sites (30,34) due to a weakening of the glycosidic bond. These abasic sites can subsequently become mutagenic upon erroneous replacement of the missing base (44). The error rate of mammalian DNA repair processes is mainly due to the intrinsic error rate of the DNA polymerases involved (thought to be $\sim 10^{-5}$). However, these polymerase induced errors may be themselves repaired during proofreading, for example, or by mismatch repair (MMR), hence the overall error rate of DNA repair may be $<10^{-5}$.

DNA glycosylases of the base excision repair (BER) pathway are involved in repairing alkylated DNA by the removal and replacement of alkylated bases (30). The BER process involves the incision of the DNA by a glycosylase, either side of the adducted base, the removal of the adducted base creating an abasic site, followed by the replacement of the base using the opposite strand as a template and finally the ligation of the newly inserted base into the DNA strand. This process is especially important for the N7 and N3 adducts of G and A, respectively. Hence, BER may contribute to DNA repair-mediated thresholds for alkylating agents that form adducts at N7G in particular, i.e. most alkylating agents.

Alkylguanine DNA transferases (AGT) also play a major role in the repair of alkylated DNA. AGT transfers the alkyl group from O^6-alkylG to an internal cysteine residue within the AGT protein, in a enzymatic suicide process (44–46). The mammalian AGT enzyme, unlike its bacterial counterpart is inefficient at repairing O^4-alkylT or O^2-alkylT (46,47). This lack of repair of the thymine adducts in mammals is reflected by their persistence *in vivo*, where their half-lives are 30 times longer than O^6-alkylG (48). Furthermore, in AGT deficient cells, ENU induces 8-fold more GC mutations in human cells due to increased persistence of O^6-ethylG (49).

Given that O^6G adducts are efficiently repaired by mammalian cells whereas thymine adducts are not, and that DNA repair may contribute to the presence of a mutation threshold, then mutation thresholds may be detectable for GC to AT mutations but less so for mutations of AT bases in cells exposed to alkylating agents. In fact, for alkylators that induce thymine adducts, the mutations at AT bases may exhibit more linear responses, than alkylators inducing GC mutations. It has been shown that at low doses ENU induces more AT mutations than GC mutations due to AGT removing O^6G, but at higher ENU doses, more GC mutations are seen due to saturation of AGT (50). This may well reflect a potential mechanism for a non-linear dose response.

In order to further understand the specific mechanisms that may contribute to genotoxic thresholds, we compare here the reactivity, adduction targets and mutagenicity of several alkylators including ENU and MMS. This takes advantage of the differing mechanisms of ENU and MMS in terms of DNA targeting and DNA repair. The DNA targeting and mutation specificities of the differing alkylators (e.g. O^6 versus N7), may allow insights into the mechanisms involved in the induction of genotoxic thresholds. In order to assess the differences between ENU and MMS in particular, in terms of DNA targeting and mutagenesis, we need to understand more about the mode of action of these chemicals.

Ethylnitrosourea

ENU was shown in 1969 to be mutagenic (51) and has since been used as a model mutagenic agent. ENU is a monofunctional agent, with a Swain Scott constant of 0.26 and an S_N1 mechanism of action. Hence, ENU is reactive with the less nucleophilic centres such as O^6G. ENU mainly targets the DNA backbone (58%), followed by N7G (11%), O^6G (8–9%), O^2T (7–8%) and O^4T (1–2.5%), as shown in Table III (34). ENU is unlike many alkylating agents in that it targets oxygens (31). It was originally thought that the nitrogen adducts of alkylators contributed only to cytotoxicity (52), however, it is now known that these nitrogen adducts are potentially mutagenic, via their conversion into abasic sites (53). ENU, due to its induction of ethylthymine adducts, induces mutations at AT bases (50). This is in contrast to all other alkylating agents that mainly target guanine and produce GC to AT mutations (31). Therefore, in terms of thresholds, it may be the case that ENU-induced AT mutations are less prone to a thresholded response than MNU (its related

methylating agent)-induced GC mutations, due to the differing repair capacities.

Methylmethanesulfonate

MMS, a solvent, insecticide and chemotherapeutic agent (54) has a high Swain Scott constant ($s > 0.83$) and functions via an S_N2 mechanism targeting adduct formation at the nucleophilic centres such as N7G. MMS differs from ENU in adduction targets as is shown in Table III. Whilst ENU readily forms O^6-alkylG, MMS does not, preferentially targeting N7G. Indeed MMS forms over 80% of its methyl adducts at N7G, with another 10% targeted at N3A. Only 0.3% of its alkylation is targeted at O^6G (34), O^4T and O^2T are not produced by MMS. MMS is a relatively weak mutagen (55,56). A reason for this may be that, as mentioned above, the predominant adduct formed by MMS (N7G), is not thought to be a mutagenic lesion itself, but gives rise to abasic sites which may be subsequently mutagenic (53). In addition, ring opened 7methylG (RO7meG) has been detected after exposure to methylating agents (34). This ring-opened adduct of N7G inhibits replication (57) and constitutes a potential mutagenic threat. As MMS induces mutations indirectly (via abasic sites and ring opened structures) it may well be prone to a thresholded response. Indeed, it is envisaged that these N7G adducts and the abasic sites produced from them are repaired by BER, hence BER may contribute to a genotoxic threshold for MMS.

Sequence specificity of ENU, MNU, EMS and MMS

To investigate the possibility that mutation thresholds exist for the alkylating agents (including ENU and MMS), sequence specificities of induced mutations were first sought for a range of alkylating agents (ENU, EMS, MNU, MMS) (Table IV and Figure 3). In addition to searching the literature, the data accumulated in the Mammalian Gene Mutation database (MGMD) (http://lisntweb.swan.ac.uk/cmgt/index.htm) (58) were used to compare the mutation profiles of these chemicals and to correlate induced mutations with the adduct types shown in Table III. Understanding the mutation profiles of the differing alkylators will help identify the different adduct types induced by the agents and hence which DNA repair pathways may be involved in their removal. This should aid in answering the question, 'do these alkylating agents display a threshold for mutation induction'?

The main system used to generate these alkylating agent mutation data was the *Hprt* mutation system which relies on recovering *hprt* mutant clones from a background of *hprt*$^+$ cells by exposure to thioguanine, which poisons *hprt* competent cells. Figure 3 shows *hprt* mutation types induced by ENU, EMS, MNU and MMS, and Table IV shows the raw data included in Figure 3. As can be seen from these figures, the mutation specificities of these chemicals differ widely. ENU induces most mutations (68%) at AT bases whereas EMS, MNU and MMS induce far fewer AT mutations (6, 7 and 19%, respectively). This mimics the thymine adduct data shown in Table III showing that ENU induces ~100 times more thymine adducts than MMS, 10 times more than MNU and far more than EMS (not detected in EMS treated DNA). In contrast to ENU, the other agents induce mostly GC to AT mutations (90, 92, 69% for EMS, MNU and MMS, respectively), perhaps as a result of the accumulation of the O^6G adduct. However, the contribution of N7G to these GC to AT mutations through misreplication past abasic sites cannot be ignored.

Table IV. HPRT mutation data taken from the MGMD for four alkylators demonstrating the differing specificities of mutation mimicking differences in adduct data shown in Table III

	Pooled data from MGMD							
	ENU (S_N1) No. muts	%	MNU (S_N1) No. muts	%	EMS ($S_N1/2$) No. muts	%	MMS (S_N2) No. muts	%
GC > AT	206	**26**	112	**92**	307	**90**	129	**69**
AT > GC	164	**21**	5	**4**	10	**3**	19	**10**
AT > TA	255	**33**	2	**2**	8	**2**	8	**4**
AT > CG	106	**14**	0	**0**	5	**1**	10	**5**
GC > TA	39	**5**	3	**2**	9	**3**	14	**7**
GC > CG	14	**2**	0	**0**	2	**1**	8	**4**
Total	784	**100**	122	**100**	341	**100**	188	**100**
Transitions		**47**		**96**		**93**		**79**
Transversions		**53**		**4**		**7**		**21**

In total, 1435 individual mutational events are included in this dataset from 25 published papers. Bold figures represent two percentage values.

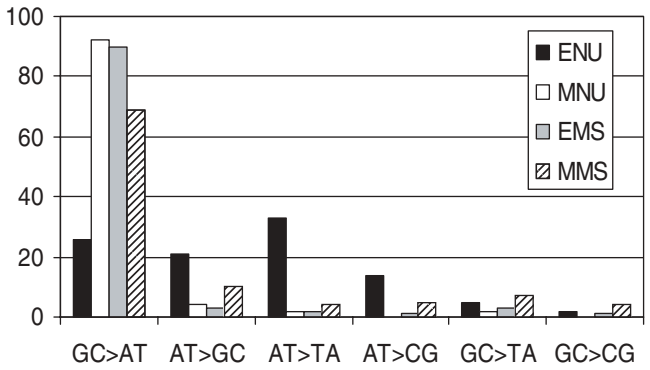

Fig. 3. Graphical representation of the data shown in Table IV, showing the differing specificities of the four alkylators in terms of mutagenicity.

Chromosome aberrations induced by alkylating agents

Most alkylating agents are direct-acting DNA reactive chemicals that have cytotoxic and mutagenic properties. They are capable of inducing a variety of lesions including DNA adducts, cross-links and strand breaks, which can be expressed as chromosomal aberrations.

Bi-functional alkylating agents tend to be more cytotoxic than the monofunctional forms, as they are capable of inducing DNA cross-links, which block DNA replication and give rise to sister chromatid exchange (SCE). Chemicals such as mitomycin C and the nitrogen mustards generate inter-strand cross-links (59,60), while cisplatin (cis-diamine dichloroplatin) is associated with intra-strand cross-links (61,62). However these cross-linking agents need the nucleophilic sites of the bases with which they can react to be appropriately positioned in each DNA strand before they can cause alkylation. As a consequence, this is a largely inefficient reaction and is relatively rare.

Both mono- and bi-functional alkylating agents produce adducted bases, the main sites targeted are N7G followed by O^6G. The O^6-alkylguanine adducts (particularly O^6-methylguanine) appear to be responsible for most ensuing mutations and chromosome modifications such as SCEs and chromosome breakage (63–66). The mechanism underlying the conversion of this adduct to chromosomal damage is as yet unclear, but potential models have been described. If the

O^6-alkylguanine adduct is not removed prior to S-phase, DNA replication can occur past the lesion, but it is mispaired with T instead of C. Mismatch repair then attempts to correct this defect by excising the mismatched T, generating strand gaps, which subsequently block the next cycle of DNA replication. The stalled replication forks may result in chromosomal aberrations by inducing double-strand breaks (67,68). In terms of N7G, BER similarly removes the adducted base and creates a temporary strand break, which is prone to leading to double-stranded breaks and chromosome fragmentation.

As previously stated, O^6G may well contribute to a threshold for alkylating agent-induced clastogenicity where saturation of repair at the threshold dose (and the LOEL dose range) allows chromosome breakage to occur. Similarly, N7G may be a thresholded lesion due to the contribution and saturation of BER. Obviously, we cannot rule out a possible role for alkylating agents in also damaging non-DNA components of chromosome segregation (e.g. spindle fibres).

Evidence for the presence of a genotoxic threshold for alkylating agents in mutagenesis/chromosome damage

Point mutation threshold

Van Zeeland *et al*. (39) have shown that the relationship between ENU exposure, the number of O^6G adducts and induced GC to AT mutations in mouse testes is non-linear, indicating a threshold effect for ENU-induced mutations. Although, in this case, the authors found that the DNA adduction step was non-linear, with the adduct to mutation step being linear.

ENU-induced O^6-alkylG accumulation and mutagenesis both exhibit thresholds in *Salmonella typhimurium* (50). As mentioned earlier, the change in mutation type between low and higher doses of ENU (AT mutations to GC mutations) also suggests the basis for a non-linear thresholded response. Indeed, the contribution of DNA repair towards a histidine revertant mutation threshold in ENU-exposed *S.typhimurium* has also been established by Sofuni *et al*. (6) who demonstrated that strains without AGT were more sensitive to alkylator-induced mutation compared with the strains proficient in AGT. Sofuni *et al*. (6) also showed that ENNG-induced revertants in the repair deficient strains at doses 10- to 100-fold lower than the doses needed to induce revertants in the repair proficient strains (0.01–0.1 and 1 μg/ml, respectively). This suggests that strains with a normal DNA repair capacity, when treated with alkylating agents, had a biological threshold below which DNA damage was repaired. However, in wild-type *S.typhimurium*, only the O^6G adduct induced by ENU exhibited a DNA lesion threshold (50). This threshold effect is likely to be due to the efficiency of DNA repair in removing this adduct from DNA, and the implication of this is that not only do thresholds have to be proved on a case-by-case basis but also on an adduct-by-adduct basis. Hence, it is entirely plausible that a particular chemical (one that induces a plethora of adduct types) can be both thresholded and non-thresholded depending upon the adduct type. This may make assigning genotoxic thresholds for chemicals very complicated, because individual thresholds for individual lesions may sometimes need to be taken into account.

There is currently very little available threshold data for adduct formation or mutation induction for MMS. However, our group has recently established that a mutation threshold exists for MMS in the *hprt* and *tk* loci of human AHH-1 cells (69). These preliminary data, shown in Figure 4, illustrate that at low doses of MMS exposure, no increase in *hprt* or *tk* mutants are observed, but at the threshold dose (~1 μg/ml), there is a sharp increase in recovered mutants. These mutations were sequenced to ensure that true DNA mutations were induced and the mutations were shown to be mostly GC to AT transitions. Furthermore, there is another published report that suggests that the dose responses of MMS to mutation induction show a shouldered (quasi-thresholded) effect in a mammalian *hprt*/*oubain* mutation system (70).

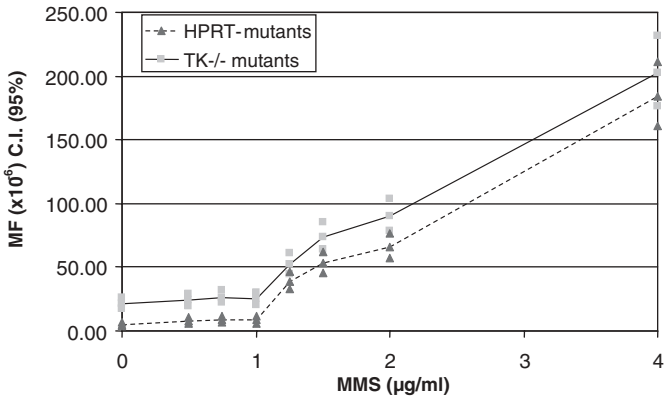

Fig. 4. *Hprt* and *tk* data demonstrating a threshold for MMS-induced mutation at 1μg/ml (69). The *Hprt* (triangles) and *tk* (squares) systems were used here in AHH-1 cells. Post-MMS treatment (24 h), mutants surviving 6-thioguanine and trifluorothymidine exposure, respectively, were counted, 14 days after MMS exposure (13 × 96-well plates per dose scored). Mutation frequency was plotted against dose along with 95% CI. Exon 3 of the *hprt* gene was PCR amplified and sequenced to confirm mutation induction; mutations were found to be GC to AT transitions, spontaneous mutations in controls were mostly deletions.

Chromosome damage thresholds

Following exposure to a range of alkylating agents, cell lines lacking the capacity for MMR were found to have significantly lower levels of chromosome damage as compared with their counterparts with a functional repair capacity (67,68). This provides further evidence to support the view that O^6-alkylguanine lesions are converted into double-stranded breaks and subsequent chromosomal aberrations via mismatch repair, and this obviously can impact on dose response relationships.

A potential threshold for MMS-induced clastogenicity (micronuclei that are negative for kinetochores) has been demonstrated using the micronucleus assay (9,16). Figure 5 shows some preliminary data obtained by our group regarding MMS-induced clastogenic thresholds, using the micronucleus assay in MMS exposed MCL-5 cells (9). In Figure 5, it is possible to see that a sharp increase in micronuclei (mostly K^-, i.e. a clastogenic threshold) exists at doses of ~0.5–1 μg/ml, similar to that of the mutation threshold (Figure 4). Note that this threshold is produced at doses inducing low levels of cytotoxicity, as measured by binucleate frequency. In the study of Elhajouji *et al*. (16) MMS similarly did not show a thresholded response for aneuploidy (kinetochore +ve micronuclei) but only for clastogenicity (kinetochore −ve micronuclei). Furthermore, MMS appeared to exhibit a linear dose response in inducing intrachromosomal rearrangements (71). This again highlights the fact that different endpoints (e.g. aneuploidy versus clastogenicity) induced by the same

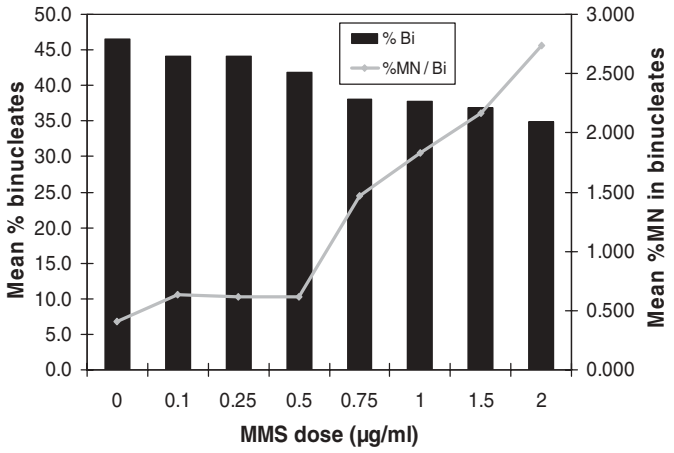

Fig. 5. Cytokinesis-blocked micronucleus data from MMS exposed MCL-5 cells (24 h exposure) showing a threshold at 0.5–1 μg/ml. One thousand binucleate cells were scored per dose for MN^+/MN^- cells. In this graph binucleate ratio (solid bars) is plotted as a measure of cell survival and MN^+ cells (line graph) are plotted as the ratio of binucleate cells against dose. The micronuclei detected here were mostly kinetochore$^-$, implying a clastogenic rather than aneugenic effect [data taken from Parry *et al*. (9)].

chemical can be inherently different in terms of the existence of a genotoxic threshold.

Conclusions

At the present time, there is a paucity of data on mutational/chromosomal threshold effects even for model DNA reactive compounds such as alkylating agents. There are a few reports which suggest that in one system or other, with one alkylator or another, mutational/chromosome damage thresholds may occur for specific alkylating agents (9,14,16,39,50,67), but convincing datasets for general effects are currently lacking. Therefore, more work is needed to ascertain if mutation thresholds can be detected experimentally. Furthermore, in terms of genotoxic thresholds, the mechanisms involved are still poorly understood, i.e. which repair pathways (if any) may be involved.

There is some evidence that O^6G-inducing alkylators exhibit thresholds of activity for mutagenicity and chromosome damage. This would correlate well with the concept that these repairable lesions could saturate the repair machinery with increasing doses and become biologically significant. Other alkylating agents inducing N7G adducts, show evidence of thresholds. If present, these thresholds must occur due to other processes, i.e. other repair mechanisms (BER, MMR, etc.), or, conjugation, exclusion, apoptosis, etc.

Current data showing thresholds for genotoxicity are only available for acute exposures to single agents. However, as most environmental exposures occur chronically, and to complex mixtures, we are a long way from fully understanding the influence of thresholded dose responses in cancer risk.

Experimental approaches that could characterize alkylating agent induced genotoxic thresholds

(i) The use of paired alkylating agents with similar/dissimilar mechanisms/adduct types. Analysing the dose response relationships of paired alkylating agents, inducing different DNA adduct profiles could allow insights into the mechanisms and targets involved.

(ii) The use of repair-deficient cell lines would allow insights into the contribution of these repair pathways to any genotoxic thresholds. Given that specific repair mechanisms may be involved in alkylator-induced genotoxic thresholds, if the limited data on alkylator-induced genotoxic thresholds were repeated in repair-deficient mammalian cells, the thresholds may disappear. If thresholds still exist in these repair-deficient cells, then mechanisms other than DNA repair must contribute to the effect.

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